IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Rice et al.	Group No.:	1648
Serial No.:	09/576,989	Atty. Docket No.:	56029-4356
Filed:	05/23/2000		
For:	HCV Variants	Examiner:	Wortman, Donna C.

DECLARATION OF DR. KERIL J. BLIGHT UNDER 37 C.F.R. §1.131

I, Dr. Keril J. Blight, declare and state as follows:

1. All of the statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true.

2. I am a co-inventor of U.S. Patent Application No. 09/576,989 for HCV Variants, filed May 23, 2000 (Patent Application).

3. I, along with co-inventor Dr. Charles M. Rice (hereinafter, "we"), conceived of, and reduced to practice the inventions claimed in the Patent Application before March 31, 2000.

4. Specifically, before March 31, 2000 we identified the adaptive mutations that are described in the Patent Application. Those adaptive mutations are referenced in the attached laboratory notebook pages and computer printouts attached as Exhibit A. The terminology used to describe the cell colonies harboring HCV comprising those mutations in Exhibit A (see A25) corresponds to the terminology used in the Patent Application (see Figure 7) as follows:

BBI	HCVrep1b/Ava.1
BBII	HCVrep1b/Ava.5
BBIII	HCVrep1b/Huh.2
BBIV	HCVrep1b/Ava.7
BBV	HCVrep1b/Ava.2
BBVI	HCVrep1b/Clone A
BBVII	HCVrep1b/Clone B

5. Because the cell colonies were G418 resistant, we expected that the resistance was conferred by HCV replicons comprising adaptive mutations, harbored by those colonies. We tested this theory by sequencing the replicons, which were amplified from cDNA reverse transcribed from RNA isolated from each of the independent G418 resistant cell clones, before March 31, 2000. That data is presented at A3-A19. We then engineered each mutation back into the HCVrep1bBartMan/AvaII backbone, as described in the Example in the Patent Application. We then transcribed RNA from each reconstructed replicon and electroporated it into naïve Huh7 cells, and compared the number of G418 resistant colonies compared to that obtained for the HCVrep1bBartMan/AvaII replicon containing wild type NS5A (see A1, for example, where it was determined that the mutation identified in clone BBI was capable of increasing the frequency of G418 resistant colonies). Based on that result, we reasonably expected that the other mutations identified would similarly confer increased frequency of G418 resistant colonies, due to increased transfection efficiency of the mutant HCV.

7. I understand that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon.

_____

Dr. Keril J. Blight

August___, 2004

Electroporation Huh7 B p60

1µg vs 10µg — WT/Ava.I
~9µg Huh7 B cellular RNA(4-1-00) — pol⁻/Ava.I
— Original (HCVrepBartMan/AvaII)
— 10µg cellular RNA only

* 10 T175's split 1:2 ~ 24hr prior to electroporation
* Procedure as always (Plated ⅓ & ⅔ on p150's. Also, removed 0.5ml of 9ml for p60)
* 4.2x10⁷ cells total — Resuspend 3ml D-PBS ⇒ ~5.5x10⁶ cells/ml
* 26hr post-electroporation add G418 at 1mg/ml
* Trypsinise p60 dishes & seed on 8 well chamber slides for IF (no G418
* Acetone fix ~ 8hr post-seeding
→ IF for NS3 (1H7) ⇒ NEGATIVE!
* Plated remainder of cells (1mg/ml on a p120. Added G418 ~16hr after seeding

⇒ The deletion is adaptive

Electroporation Huh7 (CMR) p49

1µg vs 10µg — WT / Ava.I
~9µg Huh7 B cellular RNA(4-1-00) — pol⁻ / Ava.I
— Original (HCVrepBartMan/AvaII)
— 10µg cellular RNA only

* 8 T175's split 1:2 24hr prior to electroporation
* Procedure as previously (Plated ⅓ & ⅔ on p150s Also, removed 0.5ml from 1ml for p60)
* 9.2x10⁷ cells total ⇒ Resuspend 6ml PBS ⇒ 6x10⁶ cells/EP
* 24hr post-electroporation add G418 at 0.8mg/ml

⇒ Deletion is adaptive in CMR Huh7's. In fact, more colonies are consistently observed for HCVrepBartMan & HCVrep/Ava. in CMR Huh7 cells vs Bartenschlager's.

[redacted]

Big Dye Seq

KB 486	A.1	CMR # 829	2μl
KB 487	A.1	# 862	
488	A.1	# 869	
489	A.2	# 884	
490		# 885	
491		# 1038	
492		# 1039	
493	A.15	# 1039	1μl
494		# 1038	
495		# 949	
496		# 950	
497		# 970	
498		# 982	
499		# 971	
500		# 1030	
501	A.12	# 1042	1μl
502		# 931	
503		# 1047	
504		# 936	
505		# 923	
506		# 899c	
507	A.10	# 1040	1.2μl
508		# 1047	
509		# 936	
510		# 931	
511		# 923	
512	A.13	# 1029	4μl

Clone A
~40ng PCR product

KB 574	CMR # 983	PCR # 15
575	# 1024	A.15
576	# 1022	A.15
577	# 1023	A.15
578	# 819	A.15
579	# 1030	A.15

Reamplified-Inc gel purified product
14-2-00
except KB 578 – A.2

[redacted]

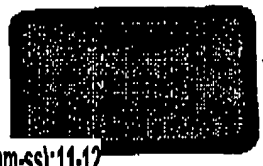
Tuesday,
Untitled

Construction parameters:

Match Size	12
Maximum Added Gap Length in Contig	70
Maximum Added Gap Length in Sequence	70
Minimum Match Percentage	65
Maximum Register Shift Difference	70
Lastgroup Considered	2
Gap Penalty	0.00
Gap Length Penalty	0.70
Consensus Threshold	75

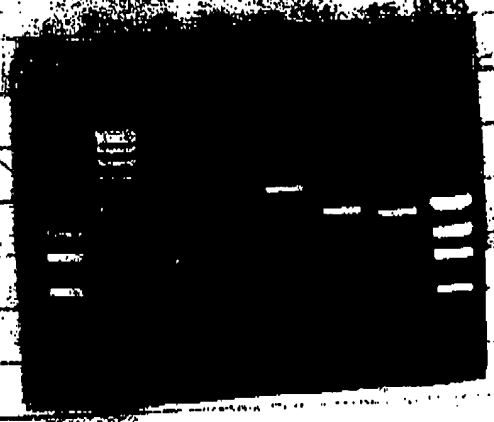
Clone A

CREATING NEW contig 1: from HartMan WT(1>8012)
ENTERING 01•KB486(12>691) in contig 1: percent match 99
ENTERING 02•KB487(33>650) in contig 1: percent match 94
ENTERING 03•KB488(36>444) in contig 1: percent match 99
ENTERING 04•KB489(18>657) in contig 1: percent match 98
ENTERING 05•KB490(26>629) in contig 1: percent match 95
ENTERING 06•KB491(1>516) in contig 1: percent match 98
ENTERING 07•KB492(8>848) in contig 1: percent match 95
ENTERING 08•KB493(1>520) in contig 1: percent match 97
ENTERING 09•KB494(2>740) in contig 1: percent match 97
ENTERING 10•KB495(1>742) in contig 1: percent match 96
ENTERING 11•KB496(21>786) in contig 1: percent match 98
ENTERING 12•KB497(20>737) in contig 1: percent match 96
ENTERING 13•KB498(4>843) in contig 1: percent match 96
ENTERING 14•KB499(1>661) in contig 1: percent match 93
ENTERING 15•KB500(11>779) in contig 1: percent match 97
ENTERING 16•KB501(1>112) in contig 1: percent match 92
ENTERING 17•KB502(17>791) in contig 1: percent match 98
ENTERING 18•KB503(1>752) in contig 1: percent match 99
ENTERING 19•KB504(2>737) in contig 1: percent match 97
ENTERING 20•KB505(21>720) in contig 1: percent match 98
ENTERING 21•KB506(8>757) in contig 1: percent match 98
ENTERING 22•KB507(1>633) in contig 1: percent match 98
ENTERING 23•KB508(1>728) in contig 1: percent match 98
ENTERING 24•KB509(7>745) in contig 1: percent match 98
ENTERING 25•KB510(18>703) in contig 1: percent match 99
ENTERING 26•KB511(1>711) in contig 1: percent match 94
Sequence 27•KB512 was not added, it is all poor data
Elapsed Time 0:0:16



Sunday,
Project: alignment (CloneA PCR1 Contig 1
Ser->Ile
BartMan WT(1>8012)
15•KB500(11>779)
KB579(16>741)
KB575(11>586)
KB577(21>334)
22•KB507(1>633)

* Separate PCR products on 0.8% LMT
* Isolate DNA from gel slices (qs on 5-200)
* Pellet purified DNA & wash 2x 80% EtOH
* Resuspend 10µl TE
* Quantitate 0.5µl



Avg 1

Big Dye Seq ~100g PCR product / 0.8µl oligo @ 4pmol/µl

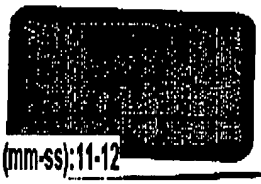
KB513	CMK #862	PCR #1	KB528	CMK #1029	PCR #3	KB571	CMK
514	#867		529	#1022	PCR #3	572	CMK
515	#829		530	#1030	PCR #3	573	CMK
516	#884	PCR #2	531	#1040	PCR #4		
517	#885		532	#1047			
518	#1038		533	#936			
519	#1039		534	#931			
520	#1038	PCR #3	535	#923			
521	#1039		536	#1047	PCR #5		
522	#949		537	#936			
523	#950		538	#931			
524	#970		539	#923			
525	#971		540	#1042			
526	#982		541	#899c			

Wednesday,
Untitled

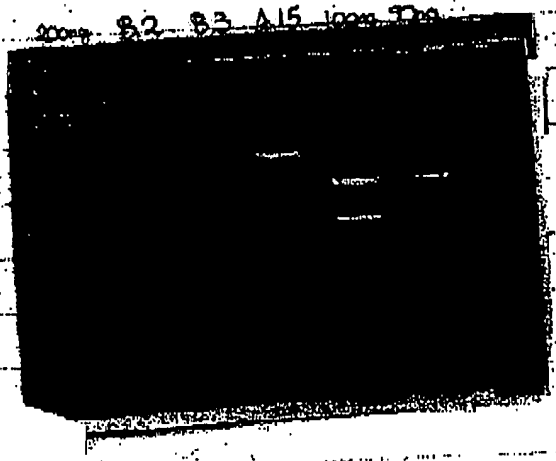
Construction parameters:	
Match Size	12
Maximum Added Gap Length in Contig	70
Maximum Added Gap Length in Sequence	70
Minimum Match Percentage	65
Maximum Register Shift Difference	70
Lastgroup Considered	2
Gap Penalty	0.00
Gap Length Penalty	0.70
Consensus Threshold	75

Ava.1

CREATING NAW contig 1: from BartMan WT copy(1>8012)
ENTERING 01•KB513(16>572) in contig 1: percent match 96
ENTERING 02•KB514(33>443) in contig 1: percent match 99
ENTERING 03•KB515(1>600) in contig 1: percent match 99
ENTERING 04•KB516(1>806) in contig 1: percent match 95
ENTERING 05•KB517(5>698) in contig 1: percent match 94
ENTERING 06•KB518(1>518) in contig 1: percent match 98
ENTERING 07•KB519(14>821) in contig 1: percent match 94
ENTERING 08•KB520(6>711) in contig 1: percent match 96
ENTERING 09•KB521(11>514) in contig 1: percent match 98
ENTERING 10•KB522(14>666) in contig 1: percent match 97
ENTERING 11•KB523(11>756) in contig 1: percent match 97
ENTERING 12•KB524(22>729) in contig 1: percent match 95
ENTERING 13•KB525(24>734) in contig 1: percent match 92
ENTERING 14•KB526(1>707) in contig 1: percent match 97
ENTERING 15•KB527(19>853) in contig 1: percent match 91
ENTERING 16•KB528(23>535) in contig 1: percent match 85
ENTERING 17•KB529(43>658) in contig 1: percent match 97
ENTERING 18•KB530(42>775) in contig 1: percent match 89
ENTERING 19•KB531(7>679) in contig 1: percent match 98
ENTERING 20•KB532(19>723) in contig 1: percent match 98
ENTERING 54•KB533(1>859) in contig 1: percent match 96
ENTERING 55•KB534(1>750) in contig 1: percent match 97
ENTERING 56•KB535(17>755) in contig 1: percent match 98
ENTERING 57•KB536(8>829) in contig 1: percent match 97
ENTERING 58•KB537(1>763) in contig 1: percent match 96
ENTERING 59•KB538(12>697) in contig 1: percent match 99
ENTERING 60•KB539(18>739) in contig 1: percent match 99
ENTERING 61•KB540(22>114) in contig 1: percent match 89
ENTERING 62•KB541(25>692) in contig 1: percent match 98
Elapsed Time 0:0:18



Project: Aval align(dsln corr.) Contig 1
5080
5090
5100
5110
5120
5130
5140
5150
5160
17-KB529(43>658)
18-KB530(42>775)
16-KB528(23>535)
5180
5190
5200
5210
5220
5230
5240
5250
5260
BartMan WT copy(1>8012)
17-KB529(43>658)
18-KB530(42>775)
16-KB528(23>535)
5270
5280
5290
5300
5310
5320
5330
5340
5350
BartMan WT copy(1>8012)
18-KB530(42>775)
16-KB528(23>535)
5360
5370
5380
5390
5400
5410
5420
5430
5440
5450
BartMan WT copy(1>8012)
18-KB530(42>775)
16-KB528(23>535)
KB571(28>194)
5460
5470
5480
5490
5500
5510
5520
5530
5540
BartMan WT copy(1>8012)
18-KB530(42>775)
16-KB528(23>535)
KB571(28>194)
5550
5560
5570
5580
5590
5600
5610
5620
5630
BartMan WT copy(1>8012)
18-KB530(42>775)
16-KB528(23>535)
KB571(28>194)
13-KB531(7>679)
Page 10



B.2 80ng/µl
B.3 80ng/µl
A.15 120ng/µl

Big Dye Seq ~ 40ng PCR product

KB542	CMR #862	B.1	KB560	CMR #1040	B.4
543	#867	B.1	561	#1041	
544	#829	B.1	562	#936	
545	#884	B.2	563	#931	
546	#886		564	#923	
547	#1038		565	#1047	B.5
548	#1039		566	#936	
549	#1038	B.3	567	#931	
550	#1039		568	#923	
551	#949		569	#1042	
552	#950		570	#899c	
553	#970				
554	#971				
555	#982				
556	#983				
557	#1029				
558	#1022				
559	#1030				

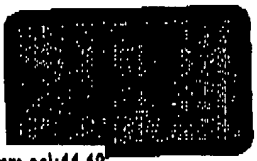
Thursday,
Untitled

Construction parameters:

Match Size	12
Maximum Added Gap Length in Contig	70
Maximum Added Gap Length in Sequence	70
Minimum Match Percentage	65
Maximum Register Shift Difference	70
Lastgroup Considered	2
Gap Penalty	0.00
Gap Length Penalty	0.70
Consensus Threshold	75

Clone B

CREATING NEW contig 1: from BartMan WT copy(1>8012)
ENTERING 31•KB542(41>556) in contig 1: percent match 99
ENTERING 32•KB543(34>444) in contig 1: percent match 99
ENTERING 33•KB544(3>597) in contig 1: percent match 99
ENTERING 34•KB545(1>690) in contig 1: percent match 95
ENTERING 35•KB546(1>598) in contig 1: percent match 96
ENTERING 36•KB547(1>515) in contig 1: percent match 97
ENTERING 37•KB548(1>574) in contig 1: percent match 95
ENTERING 38•KB549(12>678) in contig 1: percent match 98
ENTERING 39•KB550(29>512) in contig 1: percent match 98
ENTERING 40•KB551(15>620) in contig 1: percent match 97
ENTERING 41•KB552(7>676) in contig 1: percent match 98
ENTERING 42•KB553(12>694) in contig 1: percent match 98
ENTERING 43•KB554(2>597) in contig 1: percent match 93
ENTERING 44•KB555(1>696) in contig 1: percent match 98
ENTERING 45•KB556(5>707) in contig 1: percent match 96
ENTERING 46•KB557(6>567) in contig 1: percent match 95
ENTERING 47•KB558(37>700) in contig 1: percent match 96
ENTERING 48•KB559(18>596) in contig 1: percent match 96
ENTERING 49•KB560(2>646) in contig 1: percent match 97
ENTERING 50•KB561(7>662) in contig 1: percent match 97
ENTERING 51•KB562(1>677) in contig 1: percent match 98
ENTERING 52•KB563(1>693) in contig 1: percent match 97
ENTERING 53•KB564(16>725) in contig 1: percent match 97
ENTERING 54•KB565(1>695) in contig 1: percent match 98
ENTERING 55•KB566(8>687) in contig 1: percent match 98
ENTERING 56•KB567(1>600) in contig 1: percent match 99
ENTERING 57•KB568(15>732) in contig 1: percent match 97
ENTERING 58•KB569(14>127) in contig 1: percent match 85
ENTERING 59•KB570(7>686) in contig 1: percent match 98
Elapsed Time 0:0:17



Thursday,
Project: Untitled Contig 1
Page 10

Big Dye Seq ~40ng PCR product Ava.5

Ava.5	PCR #1	30ng/µl
	PCR #2	170ng/µl
	PCR #3	150ng/µl
	PCR #4	60ng/µl
	PCR #5	30ng/µl

Recalculated Added 15ng PCR cdbot, instead of 40ng	KB580	CMR #862	PCR #1
	581	#867	
	582	#829	
	583	CMR #884	PCR #2
	584	#885	
	585	#1038	
	586	#1039	
	587	#1014	
	588	CMR #1038	PCR #3
	589	#1039	
	590	#949	
	591	#950	
	592	#970	
	593	#971	
	594	#982	
	595	#983	
	596	#1029	
	597	#1022	
	598	#1030	
	599	#1023	

KB600	CMR #1040	PCR #4
601	#1047	
602	#936	
603	#931	
604	#923	
605	#1047	PCR #5
606	#936	
607	#931	
608	#933	
609	#1042	
610	#849C	

Tuesday,
Untitled

```
Construction parameters:
Match Size                                  12
Maximum Added Gap Length in Contig          70
Maximum Added Gap Length in Sequence        70
Minimum Match Percentage                    65
Maximum Register Shift Difference           70
Lastgroup Considered                        2
Gap Penalty                                 0.00
Gap Length Penalty                          0.70
Consensus Threshold                         75

CREATING NEW contig 1: from PartMan WF copy 1(1>8012)
ENTERING  01•KB580(33>533) in contig 1: percent match 97
ENTERING  02•KB581(1>439) in contig 1: percent match 94
ENTERING  03•KB582(16>598) in contig 1: percent match 90
Sequence 04•KB583 was not added, it is all poor data
Sequence 05•KB584 was not added, it is all poor data
Sequence 06•KB585 was not added, it is all poor data
    NOT ENTERING in contig 1: 07•KB586(11>488) due to percent match (43) below threshold 65
    NOT ENTERING in contig 1: 07•KB586(11>488) due to percent match (47) below threshold 65
CREATING NEW contig 2: from 07•KB586(11>488)
Sequence 08•KB587 was not added, it is all poor data
ENTERING  09•KB588(1>665) in contig 1: percent match 97
ENTERING  10•KB589(8>515) in contig 1: percent match 98
ENTERING  11•KB590(1>739) in contig 1: percent match 96
ENTERING  12•KB591(1>734) in contig 1: percent match 96
ENTERING  13•KB592(8>738) in contig 1: percent match 95
ENTERING  14•KB593(1>576) in contig 1: percent match 94
ENTERING  15•KB594(1>708) in contig 1: percent match 95
ENTERING  16•KB595(9>698) in contig 1: percent match 94
ENTERING  17•KB596(3>558) in contig 1: percent match 94
ENTERING  18•KB597(34>551) in contig 1: percent match 98
ENTERING  19•KB598(20>545) in contig 1: percent match 98
ENTERING  20•KB599(5>325) in contig 1: percent match 94
ENTERING  21•KB600(1>596) in contig 1: percent match 97
    NOT ENTERING in contig 2: 22•KB601(1>701) due to percent match (51) below threshold 65
ENTERING  22•KB601(1>701) in contig 1: percent match 96
    NOT ENTERING in contig 2: 23•KB602(1>540) due to percent match (50) below threshold 65
ENTERING  23•KB602(1>540) in contig 1: percent match 98
    NOT ENTERING in contig 2: 24•KB603(6>648) due to percent match (45) below threshold 65
ENTERING  24•KB603(6>648) in contig 1: percent match 98
ENTERING  25•KB604(13>733) in contig 1: percent match 95
    NOT ENTERING in contig 2: 26•KB605(1>735) due to percent match (51) below threshold 65
ENTERING  26•KB605(1>735) in contig 1: percent match 98
    NOT ENTERING in contig 2: 27•KB606(1>603) due to percent match (50) below threshold 65
ENTERING  27•KB606(1>603) in contig 1: percent match 99
    NOT ENTERING in contig 2: 28•KB607(1>703) due to percent match (45) below threshold 65
ENTERING  28•KB607(1>703) in contig 1: percent match 95
ENTERING  29•KB608(17>727) in contig 1: percent match 96
ENTERING  30•KB609(14>117) in contig 1: percent match 90
ENTERING  31•KB610(43>698) in contig 1: percent match 97
Elapsed Time  0:0:17
```

Ava.5

Tuesday,
Project: Ava.5 align Contig 1
Page 9
19-KB598(20>545)
BartMan WT copy 1(1>8012)
15-KB594(1>708)
18-KB597(34>551)
17-KB596(3>558)
19-KB598(20>545)
BartMan MT copy 1(1>8012)
18-KB597(34>551)
17-KB596(3>558)
19-KB598(20>545)
BartMan WT copy 1(1>8012)
17-KB596(3>558)
19-KB598(20>545)
20-KB599(5>325)
BartMan MT copy 1(1>8012)
17-KB596(3>558)
19-KB598(20>545)
20-KB599(5>325)
Arg->Gly
5410
BartMan WT copy 1(1>8012)
17-KB596(3>558)
19-KB598(20>545)
20-KB599(5>325)

Big Dye Seq ~40ng PCR product Ava. 2

Ava. 2	PCR #1	20ng/µl
	PCR #2	120ng/µl
	PCR #3	80ng/µl
	PCR #4	40ng/µl
	PCR #5	70ng/µl

KB611	CMR#862	PCR#1
612	#867	
613	#829	
* 614	#884	PCR#2
* 615	#885	
* 616	#1038	
617	#1039	
* 618	#1014	
619	#1038	PCR#3
620	#1039	
621	#949	
622	#950	
623	#970	
624	#971	
625	#982	
626	#983	
627	#1029	
628	#1022	
629	#1030	
630	#1023	

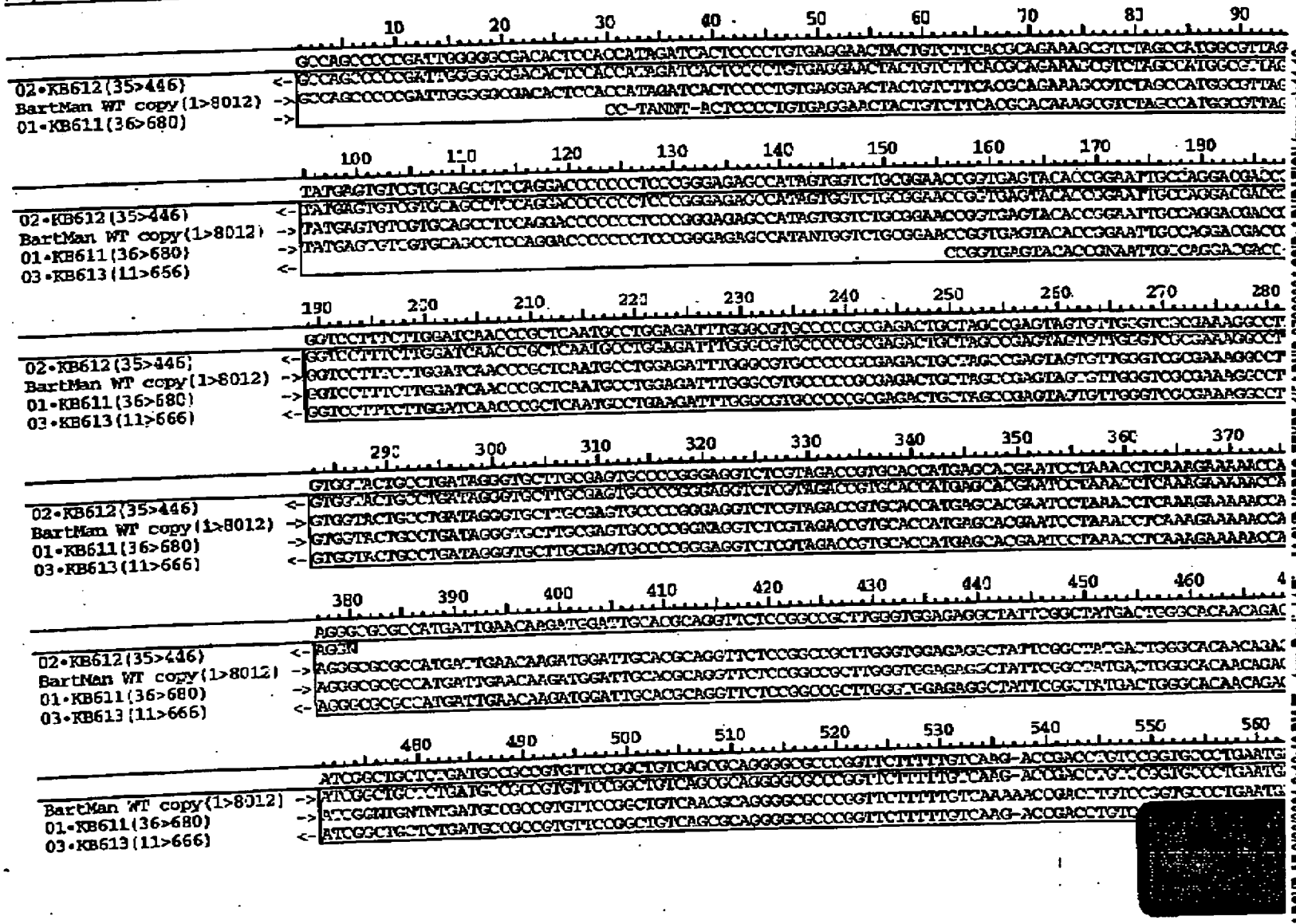
KB631	CMR#1040	PCR# 4
KB632	#1041	
KB633	#936	
KB634	#931	
KB635	#923	
KB636	#1047	PCR#5
637	#936	PCR#5
638	#931	
639	#923	
640	#1042	
641	#899c	

* Reactions 614-616 & 618 did not work → See Emily's comments

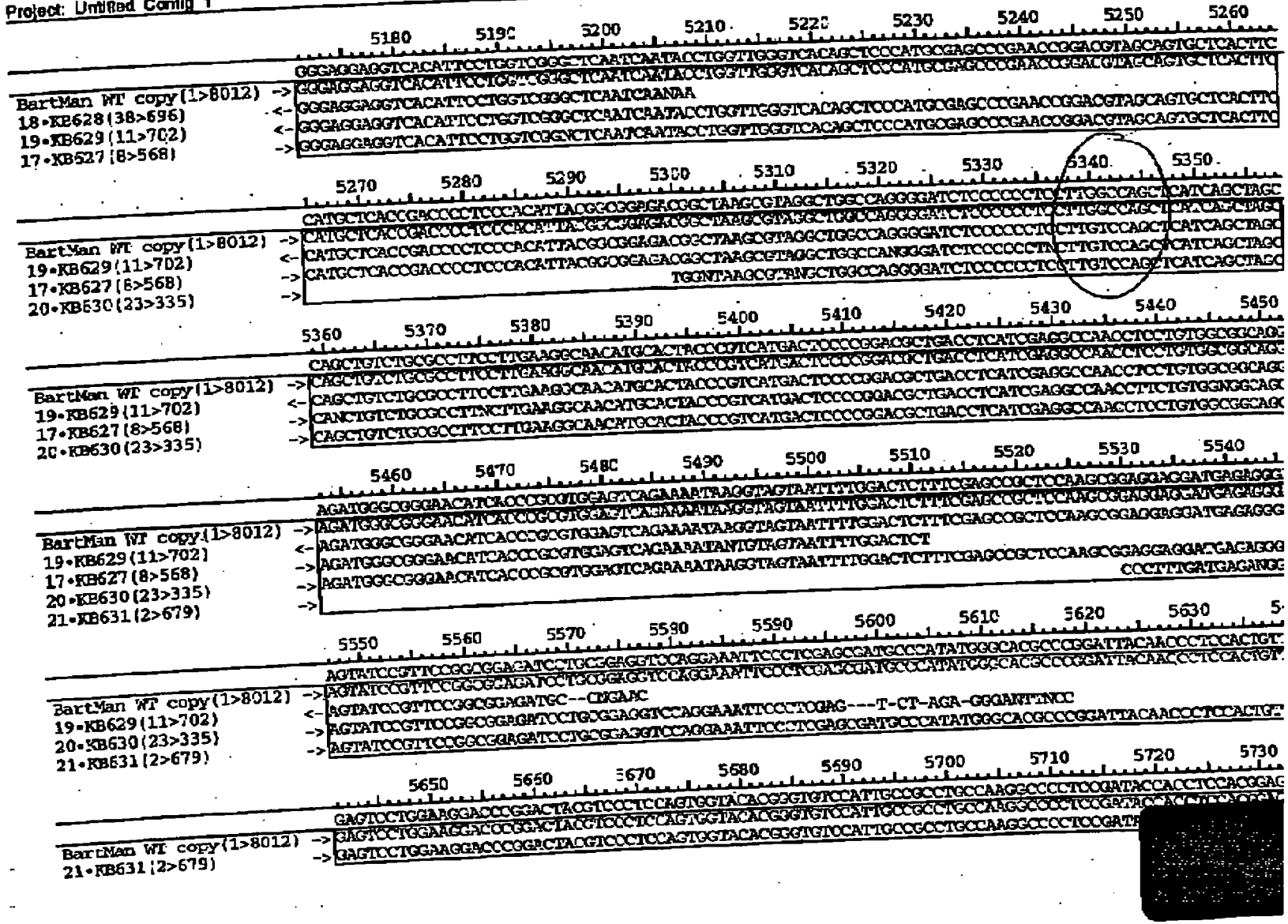
[redacted]

Ava. 2

Wednesday,
Project: Untitled Contig 1



Wednesday,
Project: Untitled Config 1



Wednesday.

Untitled

Construction parameters:

Match Size	12
Maximum Added Gap Length in Contig	70
Maximum Added Gap Length in Sequence	70
Minimum Match Percentage	65
Maximum Register Shift Difference	70
Lastgroup Considered	2
Gap Penalty	0.00
Gap Length Penalty	0.70
Consensus Threshold	75

Ava. 2

CREATING NEW contig 1: from BartMan WT copy(1>8012)
ENTERING 01•KB611(36>680) in contig 1: percent match 95
ENTERING 02•KB612(35>446) in contig 1: percent match 99
ENTERING 03•KB613(11>666) in contig 1: percent match 99
Sequence 04•KB614 was not added, it is all poor data —
Sequence 05•KB615 was not added, it is all poor data —
Sequence 06•KB616 was not added, it is all poor data —
NOT ENTERING in contig 1: 07•KB617(1>597) due to percent match (47) below threshold 65
NOT ENTERING in contig 1: 07•KB617(1>597) due to percent match (51) below threshold 65
CREATING NEW contig 2: from 07•KB617(1>597)
Sequence 08•KB618 was not added, it is all poor data
NOT ENTERING in contig 2: 09•KB619(16>662) due to percent match (45) below threshold 65
ENTERING 09•KB619(16>662) in contig 1: percent match 92
ENTERING 10•KB620(9>517) in contig 1: percent match 96
ENTERING 11•KB621(65>627) in contig 1: percent match 96
Sequence 12•KB622 was not added, it is all poor data —
ENTERING 13•KB623(38>575) in contig 1: percent match 97
ENTERING 14•KB624(6>414) in contig 1: percent match 92
ENTERING 15•KB625(1>700) in contig 1: percent match 98
TERING 16•KB626(20>542) in contig 1: percent match 95
TERING 17•KB627(8>568) in contig 1: percent match 94
ENTERING 18•KB628(38>696) in contig 1: percent match 96
ENTERING 19•KB629(11>702) in contig 1: percent match 96
ENTERING 20•KB630(23>335) in contig 1: percent match 95
ENTERING 21•KB631(2>679) in contig 1: percent match 98
NOT ENTERING in contig 2: 22•KB632(1>707) due to percent match (50) below threshold 65
ENTERING 22•KB632(1>707) in contig 1: percent match 97
NOT ENTERING in contig 2: 23•KB633(1>721) due to percent match (51) below threshold 65
ENTERING 23•KB633(1>721) in contig 1: percent match 97
NOT ENTERING in contig 2: 24•KB634(7>642) due to percent match (47) below threshold 65
ENTERING 24•KB634(7>642) in contig 1: percent match 98
ENTERING 25•KB635(13>726) in contig 1: percent match 97
NOT ENTERING in contig 2: 26•KB636(1>680) due to percent match (50) below threshold 65
ENTERING 26•KB636(1>680) in contig 1: percent match 98
NOT ENTERING in contig 2: 27•KB637(1>688) due to percent match (50) below threshold 65
ENTERING 27•KB637(1>688) in contig 1: percent match 97
NOT ENTERING in contig 2: 28•KB638(12>658) due to percent match (45) below threshold 65
ENTERING 28•KB638(12>658) in contig 1: percent match 98
ENTERING 29•KB639(15>728) in contig 1: percent match 97
ENTERING 30•KB640(8>135) in contig 1: percent match 85
ENTERING 31•KB641(1>666) in contig 1: percent match 86
Elapsed Time 0:0:16

04:30 PM

DNA Strider™ 1.3f7 ### Wednesday,

HCVrep1b BartMan/AvaII [1801 to 7758) -> Translate - 1-frame

DNA sequence 11313 bp gccagcccccGA ,?. cgactcactata circular

artenschlager replicon I377/NS3-3'UTR (Genbank AJ242652).
Constructed in the pTET backbone.
Marked by AvaII in the variable region of the 3'NTR.

→ NS3

```
1801/1
atg gcg cct att acg gcc tac tcc caa cag acg cga ggc cta ctt ggc tgc atc atc act
M  A  P  I  T  A  Y  S  Q  Q  T  R  G  L  L  G  C  I  I  T
1831/11
1861/21
agc cta aca ggc cgg gac agg aac cag gtc gag ggg gag gtc caa gtg gtc tcc acc gca
S  L  T  G  R  D  R  N  Q  V  E  G  E  V  Q  V  V  S  T  A
1891/31
1921/41
aca caa tct ttc ctg gcg acc tgc gtc aat ggc gtg tgt tgg act gtc tat cat ggt gcc
T  Q  S  F  L  A  T  C  V  N  G  V  C  W  T  V  Y  H  G  A
1951/51
1981/61
ggc tca aag acc ctt gcc ggc cca aag ggc cca atc acc caa atg tac acc aat gtg gac
G  S  K  T  L  A  G  P  K  G  P  I  T  Q  M  Y  T  N  V  D
2011/71
2041/81
cag gac ctc gtc ggc tgg cca gcg ccc ccc ggg gcg cgt tcc ttg aca cca tgc acc tgc
Q  D  L  V  G  W  Q  A  P  P  G  A  R  S  L  T  P  C  T  C
2071/91
2101/101
ggc agc tcg gac ctt tac ctg gtc acg agg cat gcc gat gtc att ccg gtg cgc cgg cgg
G  S  S  D  L  Y  L  V  T  R  H  A  D  V  I  P  V  R  R  R
2131/111
2161/121
ggc gac agc agg ggg agc cta ctc tcc ccc agg ccc gtc tcc tac ttg aag ggc tct tcg
G  D  S  R  G  S  L  L  S  P  R  P  V  S  Y  L  K  G  S  S
2191/131
2221/141
ggc ggt cca ctg ctc tgc ccc tcg ggg cac gct gtg ggc atc ttt cgg gct gcc gtg tgc
G  G  P  L  L  C  P  S  G  H  A  V  G  I  F  R  A  A  V  C
2251/151
2281/161
acc cga ggg gtt gcg aag gcg gtg gac ttt gta ccc gtg gag tct atg gaa act act atg
T  R  G  V  A  K  A  V  D  F  V  P  V  E  S  M  E  T  T  M
2311/171
2341/181
cgg tcc ccg gtc ttc acg gac aac tcg tcc cct ccg gcc gta ccg cag aca ttc cag gtg
R  S  P  V  F  T  D  N  S  S  P  P  A  V  P  Q  T  F  Q  V
2371/191
2401/201
gcc cat cta cac gcc ccc act ggt agc ggc aag agc act aag gtg ccg gct gcg tat gca
A  H  L  H  A  P  T  G  S  G  K  S  T  K  V  P  A  A  Y  A
2431/211
2461/221
gcc caa ggg tat aag gtg ctt gtc ctg aac ccg tcc gtc gcc gcc acc cta ggt ttc ggg
A  Q  G  Y  K  V  L  V  L  N  P  S  V  A  A  T  L  G  F  G
2491/231
2521/241
gcg tat atg tct aag gca cat ggt atc gac cct aac atc aga acc ggg gta agg acc atc
A  Y  M  S  K  A  H  G  I  D  P  N  I  R  T  G  V  R  T  I
2551/251
2581/261
acc acg ggt gcc ccc atc acg tac tcc acc tat ggc aag ttt ctt gcc gac ggt ggt tgc
T  T  G  A  P  I  T  Y  S  T  Y  G  K  F  L  A  D  G  G  C
2611/271
2641/281
tct ggg ggc gcc tat gac atc ata ata tgt gat gag tgc cac tca act gac tcg acc act
S  G  G  A  Y  D  I  I  I  C  D  E  C  H  S  T  D  S  T  T
2671/291
2701/301
atc ctg ggc atc ggc acg gtc ctg gac caa gcg gag acg gct gga gcg cga ctc gtc gtg
I  L  G  I  G  T  V  L  D  Q  A  E  T  A  G  A  R  L  V  V
2731/311
2761/321
ctc gcc acc gct acg cct ccg ggc tcg gtc acc gtg ccc cat cca aac atc gag gag gtg
L  A  T  A  T  P  P  G  S  V  T  V  P  H  P  N  I  E  E  V
2791/331
2821/341
gct ctg tcc agc act gga gaa atc ccc ttt tat ggc aaa gcc atc ccc atc gag acc atc
A  L  S  S  T  G  E  I  P  F  Y  G  K  A  I  P  I  E  T  I
2851/351
2881/361
aag ggg ggg agg cac ctc att ttc tgc cat tcc aag aag aaa tgt gat gag ctc gcc gcg
K  G  G  R  H  L  I  F  C  H  S  K  K  K  C  D  E  L  A  A
2911/371
2941/381
aag ctg tcc ggc ctc gga ctc aat gct gta gca tat tac cgg ggc ctt gat gta tcc gtc
K  L  S  G  L  G  L  N  A  V  A  Y  Y  R  G  L  D  V  S  V
2971/391
3001/401
atc ccg act agc gga gac gtc att gtc gta gca acg gac gct cta atg acg ggc ttt acc
```

Clone A n.t. 5336
Clone B n.t. 5336
Ant. 5345-5485
Ava. 2 n.t. 5320
Ava. 5 n.t. 3550 & n.t. 4573
n.t. 5290

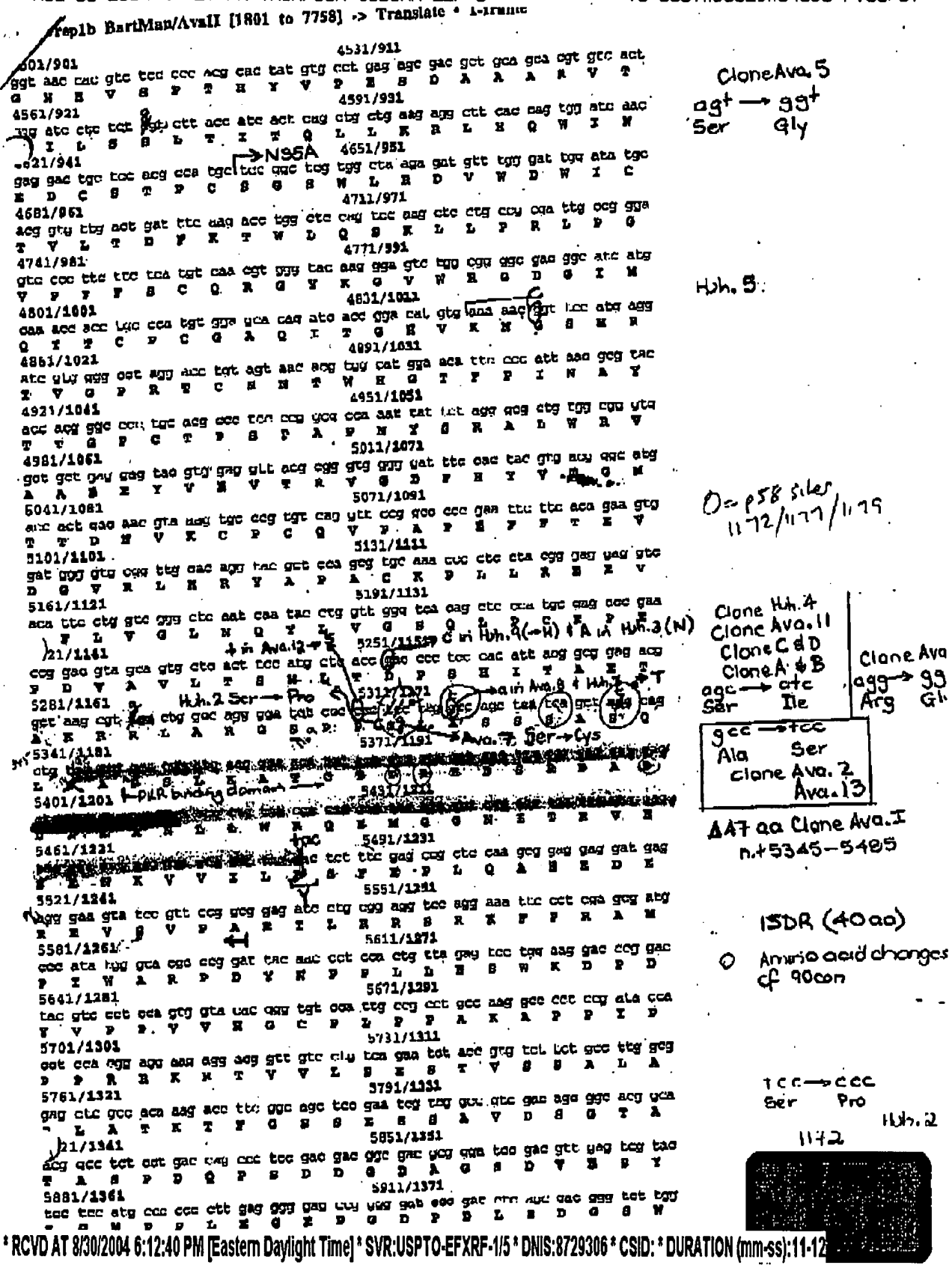
Ava. II/Clone C/Clone D
n.t. 5336

Ava. 13 n.t. 5320
Ava. 7 n.t. 5313
Huh. 2 n.t. 5314

Clone A
cag → cgg 87
Gln Arg

E → G 177
GAA GGA

T → I 255
ACC ATC


Prep1b BartMan/AvaII [1801 to 7758] -> Translate * 1-frame
NS5A
Clone Ava.5
agt → ggt
Ser Gly
Hvh. 5
Clone Hvh.4
Clone Ava.11
Clone C & D
Clone A & B
agc → atc
Ser Ile
Clone Ava
agg → gg
Arg Gl
gcc → tcc
Ala Ser
clone Ava. 2
Ava. 13
Δ47 aa Clone Ava.1
n.+5345-5405
ISDR (40aa)
0 Amino acid changes
of 90con
tcc → ccc
Ser Pro
Hvh. 2
1172

19-3-00 **G418-colonies picked for BartMan/AvaII in CMR Huh7 cells**

Huh.4	10µg	1/3	8-2-00
Huh.5	1µg	2/3	"
Huh.6	1µg	1/3	"
Huh.7	1µg	1/3	"
Huh.8	10µg	1/3	"
Huh.9	10µg	1/3	"

⇒ G418 added to 750µg/ml on 20-3-00

Huh.4

- Transfer to 24 well plate 21-3-00 p1
- Transfer to 6 well plate 22-3-00 p2
- Transfer to T25 flask 25-3-00 p3

ALSO, Cell count #1 3.2×10^5 cells/ml
Cell count #2 2.95×10^5 " } ~ 3×10^5 cells/ml

⇒ Trizol extract RNA from 80µl cells (2.4×10^4 cells)

- Transfer to T75 flask 30-3-00 p4

10-4-00 - Froze 12 vials @ 4×10^6 cells/vial Huh.4 p7 (split 1:2 8-4-00)
Tank 2 Rack 5 Box 1 (1 vial); Box 6 (1 vial); Box 7 (1 vial);
Box 8 (6 vials); Box 9 (1 vial) & Tank 2 Rack 7 Box 3 (2 vials)

Huh.5

- Transfer to 24 well plate 24-3-00 p1
- Transfer to 12 well plate 28-3-00 p2
- Transfer to 6 well plate 30-3-00 p3
- Transfer to T25 flask 2-4-00 p4

ALSO, Cell count #1 4.4×10^5 cells/ml
Cell count #2 4.15×10^5 " } 4.3×10^5

⇒ Trizol extract RNA from 80µl (~3.4×10^4 cells)

- Transfer to T75 flask -4-00 p5

20-4-00 - Froze 10 vials @ 4×10^6 cells/vial p8 (3 T175s split 1:1.5 on 11-4-00)

Huh.7

- Transfer to 24 well plate 22-3-00 p1
- Transfer to 12 well plate 25-3-00 p2
- Transfer to 6 well plate 26-3-00 p3
- Transfer to T25 flask 29-3-00 p4

ALSO, Cell count #1 2.75×10^5 cells/ml
Cell count #2 3.5×10^5 " } 3×10^5 cells/ml

⇒ Trizol extract RNA from 80µl (2.4×10^4 cells)

- Transfer to T75 flask 2-4-00 p5

12-4-00 - Froze 12 vials @ 4×10^6 cells/vial Huh.7 p8 (split 1:2 on 10-4-00)

Tank 2 Rack 7 Box 3 (2 vials)
" " Box 4 (7 vials)
" " Box 5 (3 vials)

Huh.8

- Transfer to 24 well plate 21-3-00 p1
- Transfer to 12 well plate 24-3-00 p2
- Transfer to 6 well plate 26-3-00 p3
- Transfer to T25 flask 29-3-00 p4

ALSO, Cell count #1 1.4×10^5 cells/ml
Cell count #2 2×10^5 " } $\sim 1.75 \times 10^5$ cells/ml

⇒ Trizol extract RNA from 85µl ($\sim 1.5 \times 10^4$ cells)

- Transfer to T75 flask 3-4-00 p5

14-4-00 Froze 10 vials @ 4×10^6 cells/vial Huh.8 p8 (split 1:2 12-4-00)

Tank 2 Rack 3 Box 2 (1 vial)
Box 4 (1 vial)
Box 5 (1 vial)
Box 6 (1 vial)
Tank 1 Rack 3 Box 4 (1 vial)
Box 8 (1 vial)

Remainder stored in -80°C freezer box

Huh.9

- Transfer to 24 well plate 28-3-00 p1
- Transfer to 12 well plate 6-4-00 p2 (media change 12-4-00)
- Transfer to 6 well plate 14-1-00 p3
- Transfer to T25 flask 19-4-00 p4

ALSO, Cell count #1 6.9×10^5 cells/ml } ~ 8.5×10^5 cells/ml
Cell count #2 10^6 cells/ml }

⇒ Trizol extract RNA from 70µl (~ 6×10^4 cells)

- Transfer to T75 flask 23-4-00 p5

10-5-00 - Froze 12 vials at 4×10^6 cells/vial p8
Stored -80°C freezer box

Clone D

- Transfer to 24 well plate p1
- Transfer to 12 well plate p2
- Transfer to 6 well plate p3
- Transfer to T25 flask 16-3-00 p4

ALSO, Cell count #1 3.4×10^5 cells/ml } ~ 3×10^5 cells/ml
Cell count #2 2.5×10^5 " }

⇒ Trizol extract 80µl (2.7×10^4 cells)

- Transfer to T75 flask 20-3-00 p5

31-5-00 - Froze 7 vials @ 3.6×10^6 cells/vial p8 (4 T175's split 1:2 on 29-3-00)
Tank 2 Rack 7 Box 7

[redacted]

20-4-00

Electroporation Huh7(CMR) p64

~ 6×10^6 cells/electroporation

+ 1.5µg replicon RNA + 5µg Huh7B cellular RNA (1-1-00)

(1) HCVrep/Ava.2 BB V

(2) HCVrep/Ava.5 BB II

(3) HCVrep/CloneB BB VII

(4) HCVrep/Ava.1 BB I

(5) HCVrep13/ΔISDR

(6) HCVrepBartMan/AvaII (original)

(7) HCVrep(pol-)/Ava.1

(8) Poliorep-GFP

Place electroporated cells into media. Total volume = 9.5ml

A. Plate 0.5ml / p100

B. Plate 3ml & 6ml per p150

21-4-00

• At G418 at 0.8mg/ml ~ 27hr post-electroporation

[redacted]

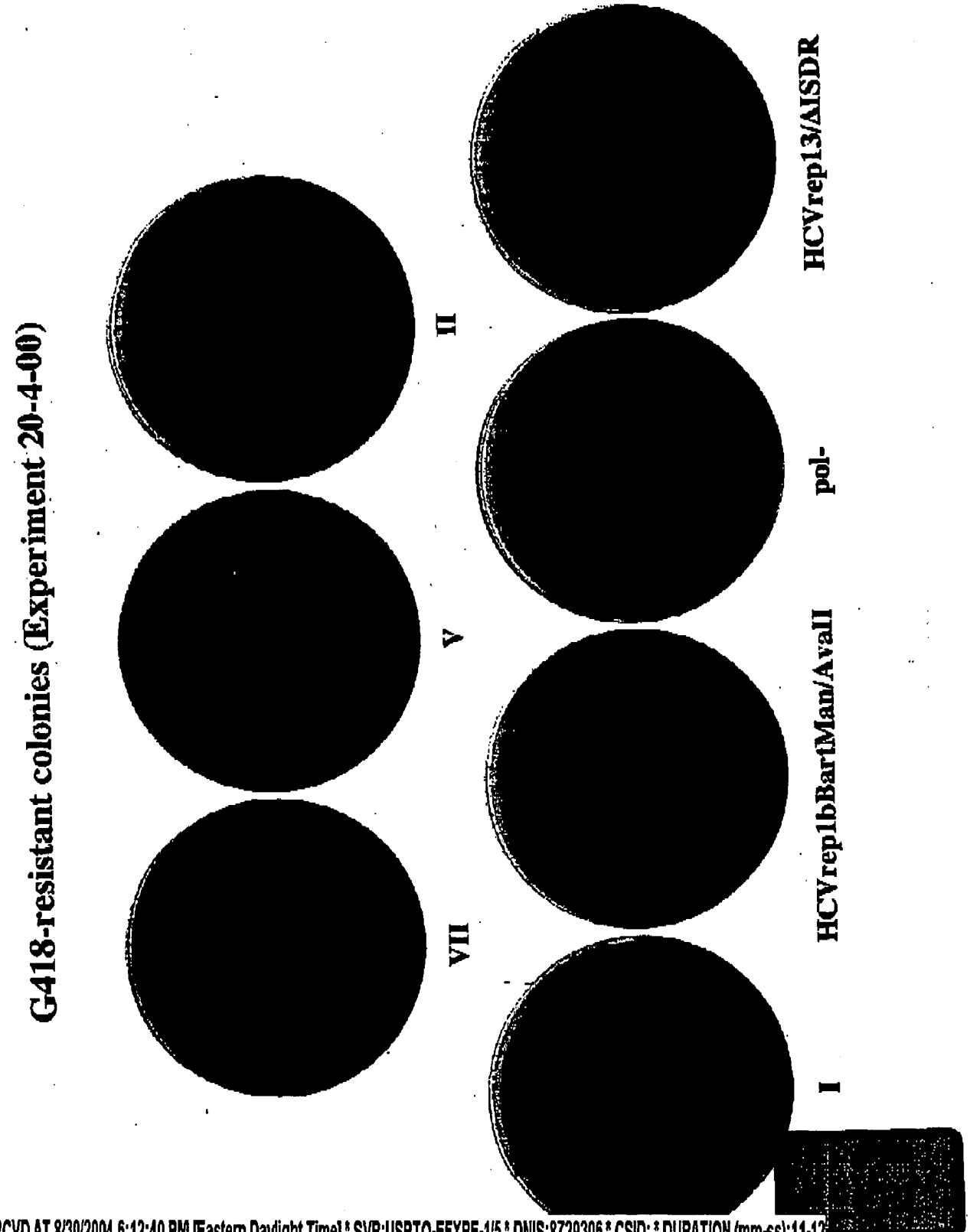
* Stain p100's on 6-5-00

* Stain p100 with 3ml plated on 14-5-00

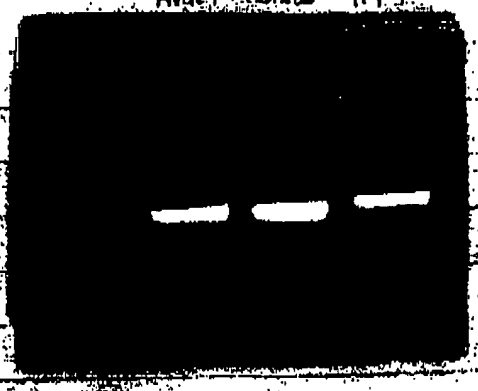
NB, When the cells are plated too dense they begin to detach due to overconfluence, particularly CloneB & Ava.2 which appear to replicate in >90% of Huh7 cells

* CloneB > Ava.2 > Ava.5 & Ava.1 # of G418-resistant colonies

* No colonies observed for H77, HCVrep13/ΔISDR & HCVrep(pol-)/Ava.1


G418-resistant colonies (Experiment 20-4-00)
I
HCVrep1bBartMan/AvaII
pol-
HCVrep13/ΔISDR
II
V
VII

Check 1µg (~2µg) on non-denaturing gel



21-6-00

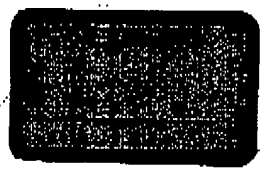
Electroporation Huh7 p69

5 T175's split 1:2 ~ 24hr prior

1.5µg HCVrep RNA + 4µg Huh7B cellular RNA (4-1-00)

~ 6×10^6 cells/electroporation

1. HCVrep1b/Ava.1 BB I
2. HCVrep1b/Ava.2 BB V
3. HCVrep1b/Ava.5 BB II
4. HCVrep1b/Ava.7 BB IV
5. HCVrep1b/Clone B BB VII
6. HCVrep1b/Huh.2 BB III
7. HCVrep1b/Ser→Ile (1179)
8. HCVrep1b BartMan/AvaII (original)
9. HCVrep1b BartMan (pol⁻)/AvaII



Volume total ~ 9.5ml → Plate 0.4ml, 0.3ml, 0.2ml, 0.1ml per p100
Plate 1.5ml per p150
(N.B. Also plated 6ml/p150 for HCVrep1b/Ser→Ile)

22-5-00

Electroporation Hela cells p15

* 5 T175's split 30hr prior 1:2
* 1.3×10^8 cells total resuspended in 8.5ml ice-cold D-PBS
* 99 µsec, 0.9 kV, 5 pulses, 0.4ml cells (~ 6×10^6 cells)
  2µg HCVrep RNA

1 HCVrep1b/Clone B BB VII
2 HCVrep1b/Ava.1 BB I
3 HCVrep1b/Ava.2 BB V
4 HCVrep1b/Ava.5 BB II
5 HCVrep1b/Ava.7 BB IV
6 HCVrep1b/Huh.2 BB III
7 HCVrep1b BartMan/AvaII
8 HCVrep1b BartMan(pol-)/AvaII
9 HCVrep13/S→I
10 HCVrep13/ΔISDR
11 No RNA

* V_T ~ 9.5ml media + EPed cells
* Plate 3ml & 6ml per p150
  " 0.5ml per p100 (For no RNA EP, plated total cells on p150)

24-5-00

At 48hr post-EP add 0.9 mg/ml G418

⇒ No colonies observed for any of the RNAs electroporated

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